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14. ABSTRACT: We proposed that decorating ovarian tumor cells with αGal (using RGD*-αGal) will lead to their destruction by patients' naturally occurring antibody against αGal. This past year we developed an in vitro assay to measure antibody-specific complement-dependent cytotoxicity (CDC) of tumors expressing αGal. We showed in mice that human serum containing anti-αGal antibody induced specific regression of αGal -expressing B16 melanoma far more than the effects against αGal non-expressing B16. In order to increase the amount of αGal that could be expressed on the surface of human tumors, we collaborated with Dr. Kiessling to modify a humanized anti-GD2 mAb by directly conjugating it with αGal. We showed this modified mAb (hu14.18K322A- αGal9) bound well to GD2+ tumors. It also delivered αGal to their membranes, as detected using an αGal -specific lectin. The hu14.18K322A- αGal9 mAb also appeared to deliver enough αGal to the surface of GD2+ tumors to enable detection of anti-Gal IgM antibody, found in human serum. However, despite the detection of αGal delivery to the surfaces of these tumor cells, we have not yet been able to reproducibly show sufficient levels of antibody and complement mediated killing to enable translation into clinical treatment simulation in vitro or in mice. These results suggest that we should pursuing means to further augment the αGal expression and the immune recognition of it by anti-Gal antibody. The studies planned for this next year in our and Dr. Kiessling's lab are jointly directed towards these goals.

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Alpha-Gal, complement-mediated killing, ovarian cancer, antitumor effect

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PROGRESS REPORT

A. Introduction

As outlined in the Statement of Work, and our last progress report, during this second year (September 15, 2009 – September 14, 2010) we have been working primarily on Aim 1 of the proposal "Characterize the mechanisms of destruction of Gal labeled tumor by anti-Gal in vitro" as well as making progress on Aim 2 "Establish murine models to enable preclinical development of RGD*-αGal".

B. Body

In studies related to Aim 1, we developed (in year 1) in vitro assays for detecting αGal on the surface of tumor cells and demonstrated specific detection of αGal^+ on mouse tumors (L5178Y lymphoma, MOSEC ovarian carcinoma) and confirmed that we found no αGal on human tumor cell lines (M21 and WM115 melanomas, OVCAR-3 and 7, SKOV3, CAOV3 ovarian carcinomas). We found it very helpful to use mouse B16 melanoma cells transfected with α1,3galactosyltransferase to express αGal (B16-αGal). This cell line was generated by our collaborator, Dr. Uri Galili (1) and was obtained from him along with the αGal^- parental B16 cell line. By having both the parental B16 and the transfected B16-αGal tumors, we have been able to use them in paired in vitro and in vivo studies addressing αGal specific recognition and destruction.

Based on results from year 1, we concluded:

- Two in vitro systems for detecting αGal on cell surface have been developed
- Two in vitro assays for complement-mediated cell cytotoxicity of αGal-expressing cells have been developed
- αGal^+ tumor cells, including MOSEC, but not αGal^- tumor cells, including OVCAR-3, are killed by human serum in vitro, indicating effective lysi, likely by anti-Gal antibody.
- In vitro treatment of αGal^- tumor cells with the RGD*-αGal compound did not result in reproducible detection of αGal on the cell surface or in complement-mediated cell killing by human serum, supporting our plans to enhance passive expression of αGal on human tumor cells via additional novel strategies.

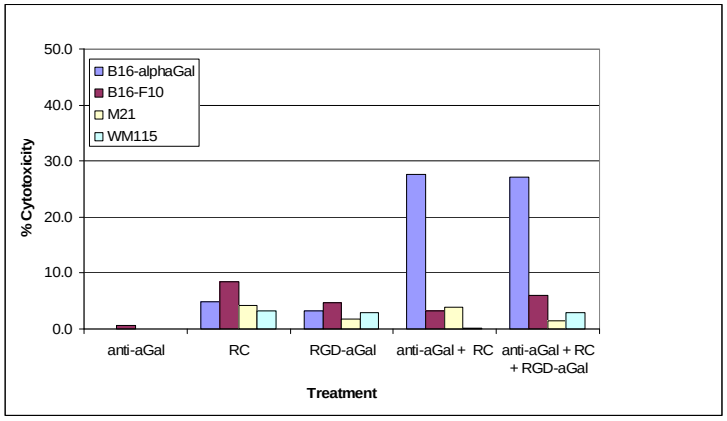

Figure 1: Complement-mediated cytotoxicity induced by anti-Gal antibody. B16-alpha Gal, B16, M21 and WM115 tumor cells were labeled with ^{51}Cr and incubated with purified anti-Gal Ab, rabbit complement (RC), RGD-aGal bifunctional ligand, or a mixture of anti-aGal Ab +RC, or a mixture of anti-aGal antibody + RC + RGD-aGal. All incubations were at 37°C for 4h. Only killing of alpha-Gal expressing tumor cells (B16-alpha Gal) was observed.

Additional analyses confirmed that the reason we didn't see augmented killing with the RGD*-αGal compound was not because of any "in vitro inhibition" by this compound. Figure 1 demonstrates that a mixture of purified anti-gal antibody + rabbit complement is able to mediate strong complement dependent cytotoxicity (CDC) (28%) on the B16- αGal tumor, with negligible background levels of CDC on the αGal-negaive B16, M21 and WM115 tumors. Importantly, the addition of the RGD*-αGal compound did not inhibit the CDC mediated by the anti-Gal antibody when cultured with rabbit complement. These data further supported our plans to develop means to augment the expression of αGal on tumors using tumor specific reagents that could passively deliver more αGal to the tumors than we had been able to achieve so far.

With this background, we have used this second year to explore, collaboratively with Dr. Kiessling, additional novel ways to enhance passive expression of αGal on the surface of human tumor cells. The division of work on this project involves the chemical synthesis and agent production to be done in Dr. Kiessling's lab, with the functional and translational extrapolation to preclinical in vitro and in vivo work to be done in our lab.

Dr. Kiessling's lab has thus been focusing on 3 separate synthetic means to create agents that enhance the amount of passively delivered αGal to the surface of tumor cells:

a. Create RGD*-αGal "dendrimers" (linking 3-5 αGal moieties to each RGD* molecule)
b. Create bifunctional ligands that link αGal to separate tumor specific small ligands (specifically the ligand for the urokinase receptor)
c. Label tumor specific antibodies with αGal.

Work underway in Dr. Kiessling's laboratory is proceeding well for all 3 of these novel approaches, and her annual report provides more detail regarding the steps involved in these synthetic process and progress for each. In brief, the RGD*-αGal "dendrimers" are nearly completely synthesized and should be ready later this fall for detailed in vitro analyses. The [Urokinase Receptor ligand - αGal] bifunctional ligands are still being synthesized, and our preclinical work with them will likely begin this winter. Of importance for this year's effort, has been the very successful creation of tumor specific monoclonal antibodies (mAbs) that have been labeled with αGal. These have been created and made available for our studies, and thus the remainder of this year's annual report is focused on this effort.

Creation of tumor specific mAbs that have been conjugated with αGal

In separate studies, our research team has been involved in the preclinical and clinical development of monoclonal antibody therapy as treatment for cancer. Our overriding hypothesis is that once a mAb binds to a tumor cell, it is important to activate immune effector mechanisms, to destroy the antibody coated tumor. These mechanisms include complement dependent cytotoxicity (CDC) and antibody dependent cell-mediated cytotoxicity (ADCC). This year our team has published 3 clinical and clinical correlative studies regarding this concept using the 14.18 anti-disialogangioside (GD2) mAb system (4, 5, 6). We hypothesized that the 14.18 mAb, which binds well to tumors that express GD2, could deliver αGal to a tumor cell surface if the mAb was directly conjugated to αGal.

As one of the mechanisms proposed for anti-αGal killing is the activation of CDC, we wished to develop an experimental model system to enable sensitive detection of anti-Gal mediated CDC. To do this we used the hu14.18K322A mutant form of the 14.18 mAb (7) . This mutated monoclonal has an amino acid switch at position 322 which abrogates the ability of the Fc end of this mAb to activate complement. In this way, we hypothesized that we could conjugate this mAb to αGal in order to specifically deliver αGal to the surface of GD2+ tumors. Once anti-Gal antibody in human serum encountered the αGal bound to the cell surface, we hypothesized that CDC would be activated. Since the unconjugated hu14.18K322A mAb can not, by itself mediate CDC, any CDC detected when the αGal conjugated mAb was bound (via the anti-GD2 mAb) to tumor in human serum, would be proof of anti-Gal induced CDC.

Thus Dr. Kiessling's lab has produced 2 separate forms of this agent: hu14.18K322A3αGal (which has ~ 3 αGal moieties per IgG molecule), and hu14.18K322A9αGal (which has ~ 9 αGal moieties per IgG molecule). Our lab has been working with both of these in our in vitro systems.

Hu14.18K322A9αGal still binds to GD2+ tumor cells.

In flow cytometry studies we have shown that conjugation of αGal to the hu14.18K322A mAb causes relatively little interference in the ability of the hu14.18K322A9αGal to bind to GD2+ tumor cells. For example, in one experiment, the background MFI (mean fluorescence intensity) units for M21 (GD2+) tumor cells was **3.2**. When these same cells were first labeled with the unconjugated hu14.18K322A mAb and then stained with a fluorescent (PE) labeled anti-human IgG detection antibody, the MFI units increased to **2043.** When this same reaction was done using the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs, the MFI values remined quite elevated, **1142** and **1154**, respectively. This result proves that the hu14.18K322A3αGal, and hu14.18K322A9αGal mAbs still retain their specific ability to bind to GD2+ tumors.

Hu14.18K322A9αGal passively deposits αGal onto GD2+ tumor cells.

Using flow cytometry, we have also demonstrated that human tumors which are negative for αGal can passively express it following treatment with these αGal conjugated mAbs. In Figure 2 we used the FITC-conjugated IB4 lectin which selectively binds αGal (2). Figure 2 shows a representative experiment using the M21 human tumor, which is GD2+ but αGal-negative. Following coating of these tumor cells with hu14.18K322A3αGal and hu14.18K322A9αGal costaining with the FITC-conjugated IB4 lectin showed MFI values of 32 and 206 respectively; both considerably greater than that seen for the controls of medium alone or medium + lectin alone. These experiments prove that the hu14.18K322A3αGal and hu14.18K322A9αGal are able to bind to the tumor and are able to deliver the αGal to the tumor surface in a manner that is detectible by the IB4 lectin.

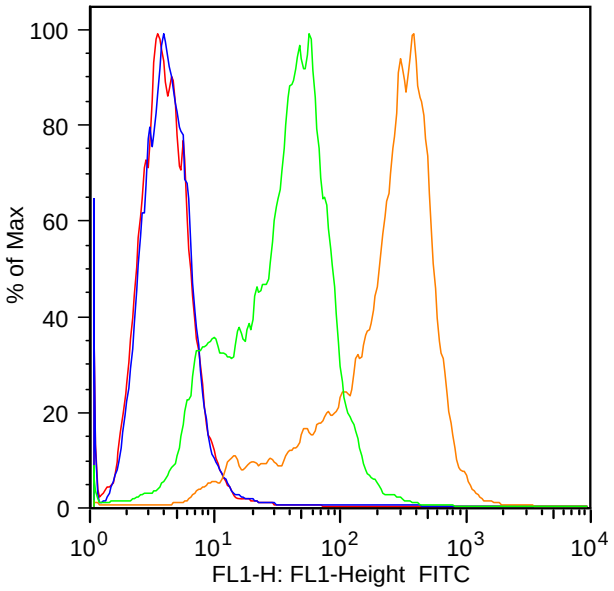

Figure 2 Legend: M21 cells ($5x10^5$ cells) were incubated with medium or hu14.18 antibody for 90 minutes at 4 C. hu14.18K322A had been modified to have either 3 molecules or 9 molecules of α-gal covalently attached to it. Cells were then washed with flow buffer. hu14.18-α gal attachment to M21 was detected by developing with fluoresceinated lectin, which binds specifically to α-gal. Fluorescence was detected using the FITC channel on a FACSCalibur flow cytometer. Histogram shows fluorescence of viable, propidium iodide-negative cells. The numbers above represent geometric means of mean fluorescence intensity (MFI) units.

In separate flow cytometry experiments, our preliminary studies demonstrate the ability of hu14.18K322A3αGal and hu14.18K322A9αGal mAbs to deliver αGal to the tumor cell surface in a manner that can be recognized by the anti-Gal antibody found in human serum. In order to perform these studies, we first incubated M21 tumor cells with hu14.18K322A3αGal and hu14.18K322A9αGal mAbs and then washed them. Next they were incubated with human serum (as a source of anti-Gal antibody) and then washed again. To determine if any of the anti-Gal antibody in the human serum actually bound to the αGal on the tumors these cells were then incubated with an anti-human IgM antibody-linked to biotin. We needed to use this anti-IgM reagent to look for any IgM anti-Gal antibody in this experimental system; we couldn't test for the presence of

any IgG anti-Gal antibody because the hu14.18K322A mAb is itself a human IgG, and is already bound to these cells. Finally, after another wash, the anti-IgM-biotin is detected by a final incubation with a strep-avidin-PE fluorescent agent, that binds selectively to the biotin. In this system we detected increased binding over background, with 163 and 173 MFI units for the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs, respectively. These preliminary data (still requiring further analyses) suggest that the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs:

a) Bind to the GD2+ tumor cells (thus, conjugating αGal to the mAb doesn't block its ability to recognize GD2)
b) Deliver αGal to the tumor surface, as recognized by lectin
c) Recruit anti-Gal antibody found in human serum (at least IgM) to the tumor surface

Do the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs induce CDC in the presence of human serum?

We then initiated testing of CDC on GD2+ tumor cells, using human serum as a source of anti-Gal antibody and also as a source of complement for CDC.

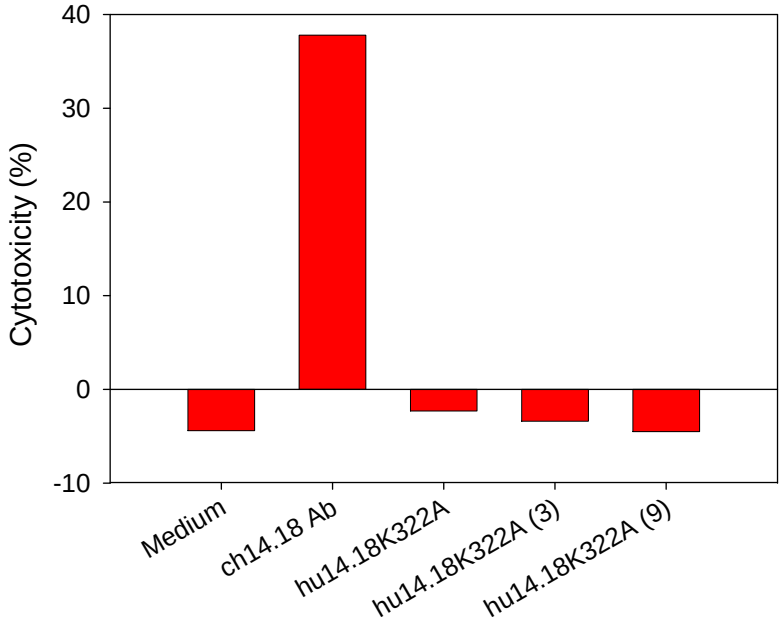

Figure 3: Complement mediated cytotoxicity of M21 cells (^{51}Cr release assay). M21 cells were labeled with ^{51}Cr in the presence of medium, ch14.18 antibody (positive control), or hu14.18K322A antibody, either intact or conjugated with 3 or 9 molecules of alpha-Gal, for at 370C for 1.5 hr. The cells were washed, and human serum added for the duration of the assay (4 hr).

Figure 3 shows a representative CDC assay done in our standard ^{51}Cr release assay system. M21 cells (GD2+, αGal –negative) were incubated with various agents, and then human serum was added as a source of anti-Gal antibody and as a source of complement. The ch14.18 anti-GD2 mAb is able to activate complement, and thus demonstrates strong killing, using the human serum as the source of complement. In contrast the hu14.18K322A mAb binds to the M21 tumor, but can't by itself activate complement, so no killing is seen. Of importance is the absence of detectible killing using the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs. We had hypothesized that the αGal on these mAbs would recruit the anti-Gal antibody in the human serum, and that this anti-Gal antibody would then activate killing by CDC using the human complement in the human serum. Repeat ^{51}Cr release assays as well as separate CDC assays using a flow-cytometry based CDC detection system (figure 4) have failed to demonstrate effective killing in human serum when GD2+ tumor cells are treated with the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs. These data suggest that additional

approaches are needed to enhance and detect anti-Gal mediated destruction induced by passive binding of αGal to tumor cells. Our plans for year 3 are outlined further below.

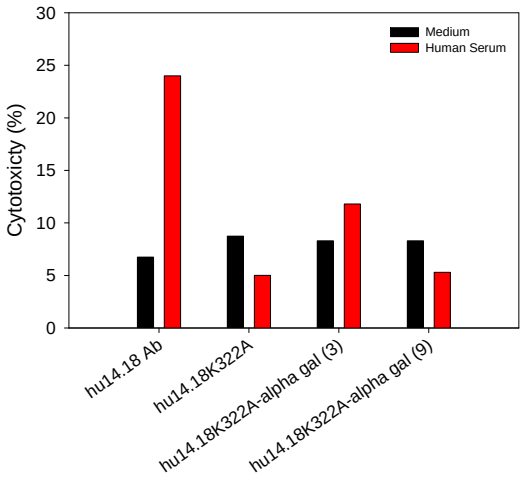

Figure 4: Complement mediated cytotoxicity of M21 cells (Flow cytometric assay). M21 cells ($3x10^5$ cells/100μL) were incubated with 1μL/ml of hu 14.18 antibody (positive control) or with hu14.18K322A antibody, either intact or conjugated with 3 or 9 molecules of alpha-Gal, for 30 minutes at 4ºC. Cells were then washed, resuspended in a 1:2 dilution of human serum (50 μL cells + 50μL serum) or medium, and incubated for 30 minutes at 4ºC followed by 60 minutes at 37ºC. Cells were labeled with propidium iodide (PI) and analyzed using a FACSCalibur flow cytometer. Percent cytotoxicty is shown on the y-axis and represents PI positive cells. While the hu14.18 mAb (able to mediate CDC) shows CDC in the presence of human serum, the mutated antibody hu14.18K322A, alone or when conjugated with alpha Gal, is not able to mediate CDC in human serum under these conditions.

In Vivo Studies:

In studies related to Aim 2 we have planned on testing the in vivo antitumor efficacy of anti-Gal antibody against tumors that can be passively labeled with αGal. We have extended our prior work by showing that human serum can show specificity against αGal-expressing tumors, in vivo. Figure 5 shows that direct intratumoral injection of human serum (containing anti-Gal antibody) has a far greater antitumor effect against the B16αGal tumor than against the parental B16 tumor. Our plans are to extend our in vitro studies to the subsequent in vivo testing of anti-Gal induced destruction of tumors that are initially αGal-negative, but can be induced to express αGal by passive administration of the agents being developed and tested in Aim 1. Until we are able to demonstrate consistent and substantial anti-Gal mediated killing in vitro, in Aim-1, against tumors passively treated to express αGal, we can not begin our proposed translation of these approaches into in vivo testing. Plans for year 3, to augment the expression of passively expressed αGal are outlined below.

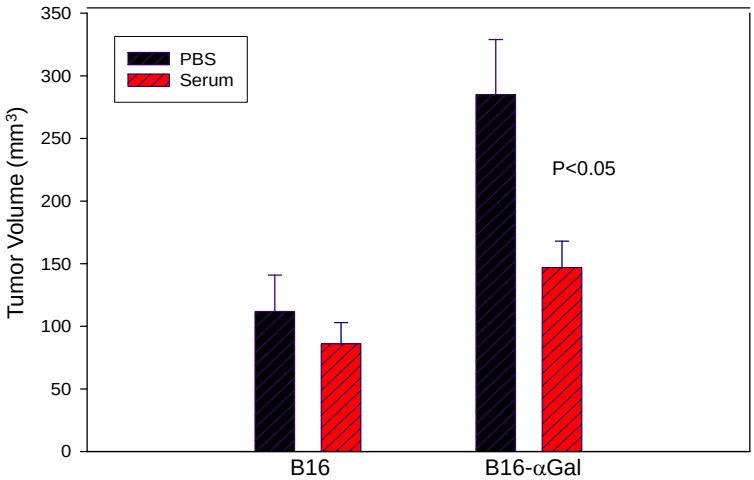

Figure 5: Antitumor effect of human serum against B16-αGal tumor. C57BL/6 mice were implanted s.c. with $1x10^5$ parental B16 melanoma cells or B16 cells expressing aGal (day 0). On day 7 and 11 the mice were injected intratumorally with 0.1 ml of either human serum or PBS (control). The tumors were measured and tumor volumes calculated. Mean SEM of 4-6 mice per group on day 15.

C. Key research accomplishments

- While RGD*-αGal can bind to tumor cells, it is not giving substantial and reproducible CDC using human serum as a source of anti-Gal antibody for tumors that do not express endogenous αGal. However, it is not inhibiting the CDC mediated by anti-Gal antibody against tumors that express endogenous αGal. Additional means are needed to augment delivery and expression of αGal by passive delivery.
- Two separate reagents have been developed that have conjugated αGal to a mAb that itself binds GD2 but doesn't activate complement: these are the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs.
- The hu14.18K322A3αGal and hu14.18K322A9αGal mAbs both bind to GD2+ tumors and passively express αGal on the tumor surface.
- Even so, GD2+ tumor cells labeled with hu14.18K322A3αGal and hu14.18K322A9αGal mAbs in vitro do not demonstrate CDC when human serum is used as a source of anti-Gal antibody and as a source of complement.

D. Reportable outcomes

- One manuscript, a review article regarding the overall rationale for anti-Gal treatment was published during this first year.

Galili U, Albertini MR, Sondel PM, Wigglesworth K, Sullivan M, Whalen G. In Situ Conversion of Melanoma Lesions into Autologous Vaccine by Intratumoral Injections of a-gal Glycolipids. Cancers, 2:773-793, 2010 (on line www.mdpi.com/journal/cancers doi:10.3390/cancers2020773)

E. Conclusions and next steps

- The conclusions from this past year of research activity are summarized above in section " **C. Key research accomplishments**".
- These accomplishments allow us to conclude that we are in fact able to deliver αGal to the surface of tumor cells by passive delivery, as proposed. However we have been unable to detect immune mediated tumor destruction at a level that would warrant proceeding to in vivo translation in preclinical mouse models.
- As CDC requires far more antibody bound to the tumor target than ADCC, we will be testing the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs together with human serum as a source of anti-Gal antibody, as well as with purified anti-Gal antibody, for their ability to induce ADCC using resting and activated human NK cells.
- In addition, as human serum is not a strong source of complement, we will test the hu14.18K322A3αGal and hu14.18K322A9αGal mAbs together with human serum as a source of anti-Gal antibody, as well as with rabbit serum as a stronger source of complement, for their ability to induce anti-Gal mediated CDC.
- Once the RGD*-αGal "dendrimers" and the [Urokinase Receptor-Ligand-αGal] reagents are available we will test them in CDC assays with human serum as a source of anti-Gal antibody and as a source of complement, and also supplement these with rabbit serum as an additional source of complement.
- Once the RGD*–αGal "dendrimers" and the Urokinase receptor-Ligand–αGal reagents are available we will also test them in ADCC assays with human serum as a source of anti-Gal antibody for their ability to induce ADCC using resting and activated human NK cells.
- As we are trying to maximize the amount of αGal that we can passively express on tumor cells, we will also combine the hu14.18K322A9αGal mAb with the RGD*-αGal "dendrimers" and the [Urokinase Receptor-Ligand-αGal], all in the same cultures, and test these in the presence of

human serum, with rabbit complement in CDC assays, and with resting and activated human NK cells in ADCC assays.

- In a related ongoing project that is conceptually linked to this preclinical project with Drs. Kiessling and Galili, we are working with Dr. Galili and our UW colleague Dr. Mark Albertini on a separate clinical trial of therapy here at the UWCCC. This clinical trial (A Phase I Study to Evaluate the Toxicity and Feasibility of Intratumoral Injection of αGal Gylycolipids in Patients with Advanced Melanoma) is approved by the FDA, open at the University of Massachusetts, and has now opened for treatment here at UW-Madison, with 2 patients enrolled. It is providing the clinical pathway for clinical treatment and monitoring of anti-Gal directed immunotherapy, that would be used for further development of the anti-Gal preclinical work being pursued in this Department of Defense project with Dr. Kiessling.

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